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56. Toxicity of the Lampricide 3-Trifluoromethyl-4-nitrophenol (TFM) to 10 Species of Algae

57. Acute Toxicities of 3-Trifluoromethyl-4-nitrophenol (TFM) and 2', 5-Dichloro-4'-nitrosalicylanilide (Bayer 73) to Larvae of the Midge Chironomus tentans

58. Acute Toxicity of the Lampricide 3-Trifluoromethyl-4-nitrophenol (TFM) to Nymphs of Mayflies (Hexagenia sp.)

59. Toxicity and Residue Dynamics of the Lampricide 3-Trifluoromethyl-4-nitrophenol (TFM) in Aquatic Invertebrates

United States Department of the Interior
Fish and Wildlife Service

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FOREWORD

The lampricide, 3-trifluoromethyl-4-nitrophenol (TFM), has been used extensively to control larvae of the sea lamprey (Petromyzon marinus) in the Great Lakes. While the toxicity of TFM to lamprey is well documented (Investigations in Fish Control, number 44), its effects and those of mixtures of TFM with Bayer 73 (2-aminoethanol salt of 2'5-dichloro-4'-nitrosalicylanilide) on other organisms are unknown. The toxicity of Bayer 73 to fish was reported in IFC, number 19.

A petition for the registration of TFM must include data describing its effects on the non-target biota and on its fate within treated animals and in the environment. Studies were conducted at the Fish Control Laboratory, La Crosse, Wisconsin and under contract by researchers at other laboratories to develop the necessary data.

The following papers concern the effects of TFM on selected species of algae and of TFM, Bayer 73, and/or mixtures of the two on selected invertebrates under laboratory conditions. These papers represent the first of a continuing series to be published in Investigations In Fish Control. All will be used to support petitions for registration to permit the continued use of lampricides. Subsequent papers in the IFC series will concern the toxicity of TFM to fishes, invertebrates, and macrophytes, the efficacy of various TFM formulations, the residue patterns associated with applications, and the biotransformation of TFM in fish and invertebrates.

Fred P. Meyer, Director
Fish Control Laboratories

INVESTIGATIONS IN FISH CONTROL

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TOXICITY OF THE LAMPRICIDE 3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM) TO 10 SPECIES OF ALGAE

By Alan W. Maki, Lester D. Geissel, and Howard E. Johnson
Department of Fisheries and Wildlife
Pesticide Research Center, Michigan State University
East Lansing, Michigan

ABSTRACT

The toxicity of analytical and field grades of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) to unialgal cultures of four green algae, four blue-green algae, and two species of diatoms was examined in 96-h toxicity tests. Growth was measured by daily optical density readings, cell counts of nonfilamentous species, and a gravimetric determination of maximum standing crop at the end of the tests. A 50% inhibition of growth (calculated from optical density data) occurred at concentrations less than 10 mg/l of TFM for all species tested except Chlorella pyrenoidosa, which was resistant at concentrations greater than 15 mg/l. Inhibition of growth was lowest in the blue-green species (50% effect levels, 9-10 mg/l), generally intermediate in the green algae, and severe in diatoms (50% effect levels, 1-4 mg/l). Field grade TFM tended to be more toxic than the analytical grade. Similar toxicity relationships were indicated by each of the three types of measurements made.

Growth tests conducted with high concentrations of TFM and subsequent filtration of the cells and resuspension in toxicant-free medium indicated that exposure to TFM at concentrations of 30 mg/l for 96 h did not destroy the viability of algal cells but temporarily inhibited growth.

INTRODUCTION

The toxicity of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) to representatives of most animal groups has been examined in several investigations (see Schnick 1972 for a review), but knowledge of the effects of the toxicant on nontarget plant and algal species is limited (Howell 1966; Haas 1970). The compound is known to be phytotoxic and has been used at relatively high concentrations for the control of rooted aquatic plants (Josephs 1961). It is necessary to determine the effects of TFM on a variety of algal species, for it is well understood that any changes in these primary producers will influence each successive trophic level in the aquatic community.

This investigation was designed to determine the 96-h toxicity of analytical and field grade TFM to unialgal cultures and to determine the effects of TFM on growth and production of selected diatoms and green and blue-green algae. Growth was measured by making daily optical density readings and cell counts of nonfilamentous species, and determining maximum specific growth rates and total biomass produced by each culture.

METHODS

Unialgal cultures of four green algae, four blue-green algae, and two species of diatoms were obtained from the sources listed in Table 1. Several culture media were evaluated

Table 1. Scientific names and sources of algal species

Species	Source
Chlorophyta	
Scenedesmus quadricauda (Turp.) Breb.	Indiana Culture Collection, Indiana University
Stigeoclonium tenue Kutz.	Indiana Culture Collection, Indiana University
Selenastrum capricornutum Printz	National Eutrophication Research Program-EPA
Chlorella pyrenoidosa Chick	Indiana Culture Collection, Indiana University
Cyanophyta	
Cylindrospermum sp.	Indiana Culture Collection, Indiana University
Anabaena flos-aquae (Lyngb.) De Brebisson	National Eutrophication Research Program-EPA
Nostoc linckia (Roth) Born. et Flash.	Michigan State University Culture Collection
Anabaena cylindrica Lemmerman	Michigan State University Culture Collection
Chrysophyta	
Nitzschia sp.	Indiana Culture Collection, Indiana University
Navicula pelliculosa (Breb.) Hilse	Indiana Culture Collection, Indiana University

and the three that produced best growth of the three algal groups were used: Green algae were grown in ASM-1 medium (Gorham et al. 1964; also in Eberly 1967), blue-green algae in Allen and Arnon medium (Allen and Arnon 1955), and diatoms in the National Eutrophication Research Program medium (Environmental Protection Agency [EPA] 1971) with the addition of silica (10 mg/l). The chemical composition of each medium is shown in Table 2. The pH of each medium was adjusted to 7.5 $\pm$ 0.2 by the addition of 0.1N HCl or 0.1N NaOH. Stock cultures were transferred to fresh media weekly to insure a

Table 2. Chemical composition of synthetic algal nutrient media

Element	Concentration in culture (mg/ℓ-major; µg/ℓ-minor)		
	NERP[a] with silica	Allen and Arnon (1955)	ASM-1
Major			
N	4.200	0.270	4.753
P	0.186	7.790	6.201
Mg	2.904	3.081	7.080
S	1.911	4.353	4.742
C	2.143	1.155	2.384
Ca	1.202	2.559	8.073
Na	11.001	12.735	76.970
K	0.469	20.735	7.812
Cl	6.611	23.585	96.465
Si	10.00	--	--
Minor			
B	32.5	62.5	431.0
Mn	115.374	62.4	333.0
Zn	15.691	6.3	210.0
Co	0.354	1.2	3.71
Cu	0.004	2.5	0.372
Mo	2.878	15.0	67.0
Fe	33.051	483.0	188.0
V	--	1.30	--

[a] National Eutrophication Research Program.

continuous supply of cells in logarithmic growth phase to serve as inoculum for the toxicity tests.

Procedures and statistical analysis of the data followed the basic recommendations of the National Eutrophication Research Program (EPA 1971). All toxicity tests were carried out in a walk-in environmental chamber where incubation conditions were constant throughout the experiments. Cultures were maintained at 23 ± 1 C under continuous, cool-white fluorescent lighting of 400 (± 10%) foot-candles. The inoculum for each test was adjusted to yield an initial cell concentration of about 1.0×10^4 cells/ml. Tests were conducted in 250-ml polyurethane-stoppered Erlenmeyer flasks with 60 ml of autoclaved medium on reciprocating shakers operating at 80 oscillations per minute.

Preliminary tests to define the range of toxicity for several species established the 50% effect at 5 to 10 mg/l; therefore, concentrations of 2.5, 5.0, 7.0, 8.0, 10.0, and 15.0 mg/l and a control were used for each toxicity test. Field grade and analytical grade TFM were tested simultaneously, with three replicates for each toxicant concentration; thus 42 culture flasks were used for each test. All data presented represent an average of two or three of these toxicity tests.

Purified TFM(A) (95% Aldrich lot #060217) and field grade (#1414, 35.7%, Drum 279 of Batch 6) TFM were used in all tests. Concentrations were based on active TFM rather than on the formulation. Primary and secondary stock solutions of the toxicant were made up in acetone and water, respectively, and periodically renewed throughout the experiments. All test flasks received 7.8 mg acetone per 60 ml culture (0.130 gm/l) from the stock solutions. Preliminary tests with three times this acetone concentration produced no measurable effects on algal growth.

Each toxicity test was conducted for 96 h to establish the lethal concentrations. The growth of the algae was determined spectrophotometrically at 680 nm on a Beckman DB spectrophotometer or a Bausch and Lomb Spectronic 20. The unicellular forms were counted directly under a microscope with a hemacytometer.

The specific growth rate as defined by the following formula was calculated for all concentrations of each toxicity test.

$$u = \frac{l_n\ (X_2/X_1)}{t_2 - t_1} \quad \text{days}^{-1}$$

where X_2 = biomass concentration at end of selected time interval

X_1 = biomass concentration at beginning of selected time interval

$t_2 - t_1$ = elapsed time in days between selected determinations of biomass (EPA 1971).

We calculated linear regressions of the effects of both analytical and field grade TFM on maximum standing crop of algae, employing the following function:

dry weight, $y = a + b$ (TFM concentration)

Biomass was expressed in milligrams dry weight of each culture.

Maximum standing crop was determined gravimetrically for each test flask at the end of the 96-h toxicity test by filtering a measured portion of algal suspension through a tared Millipore ® filter type AA with 0.80-μm pores. Filters were oven-dried at 80 C for 24 h, cooled in a desiccator, and weighed. A correction factor was developed and subtracted to correct for loss of weight of the filters during washing. Toxicant concentration was checked at the beginning and end of each toxicity test by comparison with a standard curve on a Klett-Summerson colorimeter. Concentrations of TFM in the test solutions and pH at the termination of the test were checked on a portion of the medium filtered free of algal cells.

Slight decreases in TFM concentration were observed at the termination of each test, presumably due to absorption and uptake of TFM

by algal cells. Changes in pH between initiation and termination of the test were of the order of 0.1-0.4 pH units and were not consistently different between media.

The optical density data were analyzed by the methods of Litchfield and Wilcoxon (1949) for the evaluation of median effective concentrations (EC50) and establishment of 95% confidence limits. The EC50 is the TFM concentration that causes a 50% inhibition of algal growth when compared with control cultures growing simultaneously in the absence of the lampricide.

RESULTS

Toxicity Tests

The inhibition of growth by TFM differed for the different algal taxa (Table 3; Fig. 1). In general, it was lowest for the blue-green algae, intermediate for the green algae, and most severe for diatoms (Fig. 2). Exceptions were the unicellular green alga Chlorella pyrenoidosa, which appeared to be very resistant to TFM (EC50 greater than 15 mg/l of TFM) and the filamentous blue-green algae Anabaena flos-aquae and Anabaena cylindrica, which were highly susceptible to the lampricide (EC50's, 1.8 to 4.7 mg/l of TFM). Although the EC50 values were slightly lower for field grade than for analytical TFM for each species tested, the differences were statistically significant only for the highly sensitive diatom Nitzschia sp. However, the data suggest that the additive N, N-dimethylformamide in field grade material may augment the toxicity of the compound.

Specific Growth Rates

The maximum specific growth rate (μmax--see Table 4) for individual species and concentrations at any time during incubation occurred on the second or third day of each toxicity test.

The values reflect the toxicity data presented in Table 3; the growth rates were generally best in control cultures and gradually declined as TFM concentration increased. Growth rates were more severely depressed in cultures with field grade than with analytical grade TFM. Negative growth rates for the diatoms Nitzschia sp. and Navicula pelliculosa indicate mortality of the inoculum at the higher dose levels on the second day of exposure.

In several of the cultures in 2.5 mg/l of analytical grade TFM, growth was apparently stimulated initially, since the μmax exceeded that of the controls. However, the cell density and standing crop at the end of the 4-day toxicity test were lower than in the controls, indicating that growth was suppressed at these low concentrations.

Regression analysis of toxicant concentration and maximum standing crop

The maximum standing crop for each test is defined as the weight of the total algal biomass at the end of the 4-day toxicity test, after filtration through a tared Millipore filter and oven-drying at 80 C for 24 h. Linear regressions of the effects of analytical and field grade TFM on maximum standing crop of algae were produced for the species whose growth was not severely limited by relatively low TFM concentrations.

The equations serve to establish a degree of linearity of response to the proportional increases in toxicant concentration (EPA 1971).

The linear relation between TFM concentration and maximum standing crop describes the effect of increasing TFM concentration, as evidenced by the relatively high regression coefficients for most species (Table 5). However, for species whose growth is severely limited by relatively low concentrations of the lampricide, a curvilinear relation may best describe the data. The gravimetric determination of growth (Table 5) is not as sensitive a measure of growth as is the optical density reading (Fig. 1)--for even though the optical density data indicate measurable growth, the gravimetric data do not.

Table 3. Growth inhibition of TFM[a] to algae as measured by optical density

Species	96-h EC50 and 95% confidence interval (mg/ℓ)	
	Field grade	Analytical grade
Chlorophyta		
Scenedesmus quadricauda	4.2 2.21-7.98	4.5 2.81-7.20
Stigeoclonium tenue	4.7 3.24-6.81	6.2 4.43-8.68
Selenastrum capricornutum	5.5 4.14-7.32	8.6 5.93-12.5
Chlorella pyrenoidosa	>15	>15
Cyanophyta		
Cylindrospermum sp.	9.0 7.50-10.8	9.9 3.96-24.8
Anabaena flos-aquae	3.6 2.40-5.40	4.2 2.47-7.14
Nostoc linckia	9.2 8.00-10.6	9.8 6.12-15.7
Anabaena cylindrica	1.8 0.82-3.96	4.7 2.90-7.61
Chrysophyta		
Nitzschia sp.	1.2 0.71-2.04	3.6 2.57-5.04
Navicula pelliculosa	3.2 2.00-5.12	5.2 3.71-7.28

[a] Concentration based on active TFM.

The higher toxicity of the field formulations is again shown for all three groups of algae by the lower y-intercept and depressed regression of the total biomass produced in the field grade toxicity tests. The most sensitive species are distinguished by their high intercept values and relatively shallow slopes.

Cell Counts

A hemacytometer was employed for direct microscopic counting of Scenedesmus quadricauda, Selenastrum capricornutum, and Chlorella pyrenoidosa. Accurate counts of the other species were not possible. Filamentous

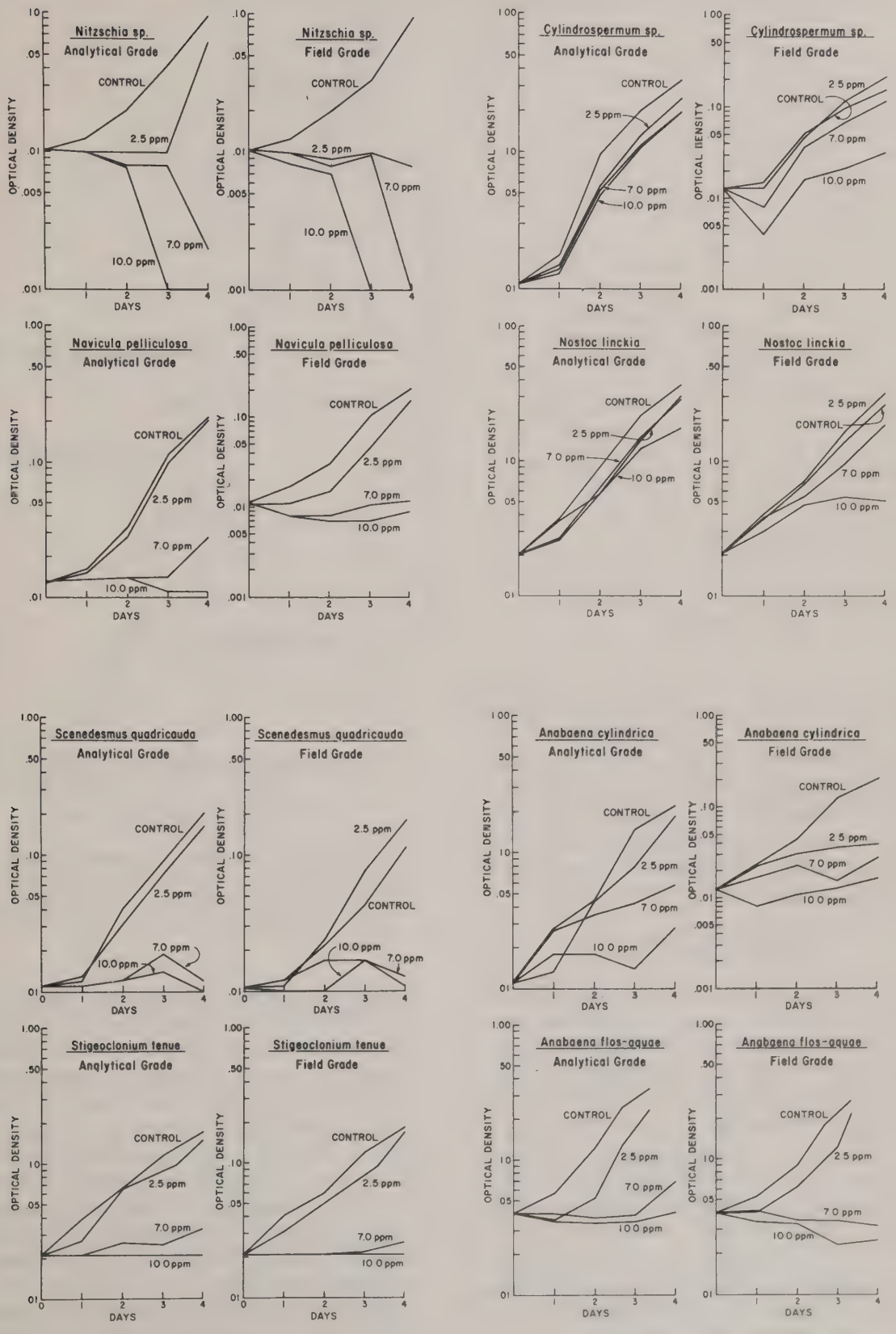

Fig. 1. Effects of analytical and field grade TFM on growth of algal cultures, measured as optical density.

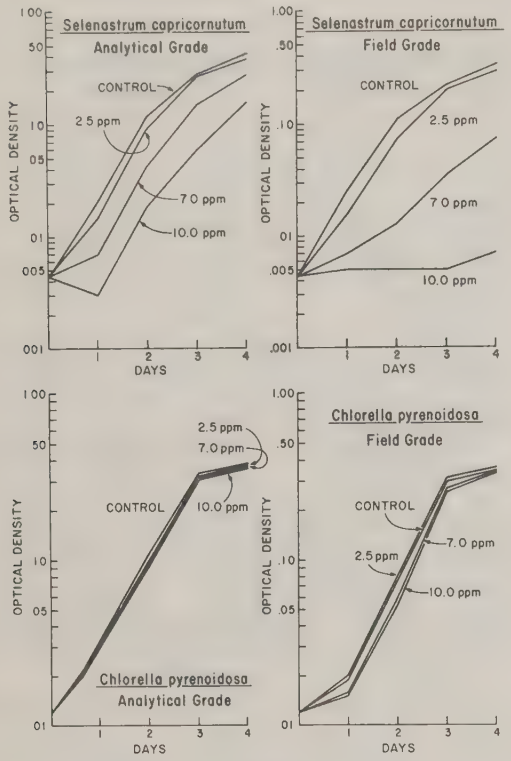

Fig. 1. Continued

species were difficult to count, and the diatoms tended to grow in small, tightly packed clumps. Attempts to equally disseminate the filamentous forms and clumping species by blending or dispelling them through a syringe were unsuccessful. The counts for the three species yielded an excellent representation of the inhibition of growth by TFM (Fig. 3).

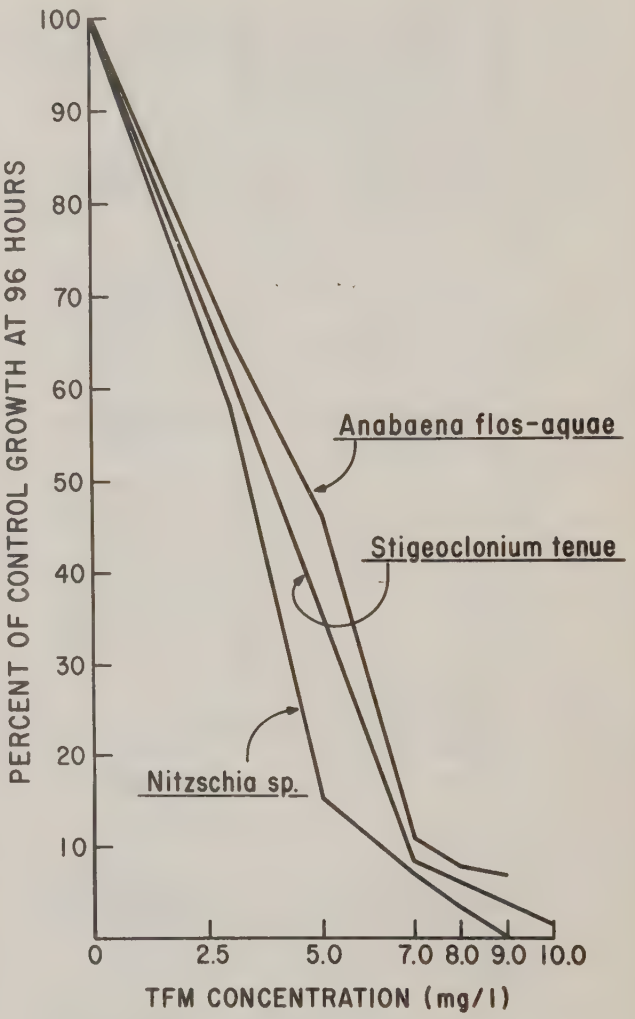

Fig. 2. Relative growth of representative algal species grown in increasing concentrations of analytical grade TFM, expressed as a percentage of control optical density at 96 h.

Mortality Tests

Inasmuch as the toxicity tests showed a definite suppression of algal growth that was directly correlated with TFM concentration, we designed a series of experiments to determine whether the growth suppression was a result of (1) toxicant-induced mortality of algal cells, (2) a temporary growth suppression of the cells at the end of the 4-day toxicity test, or (3) a selective elimination of a proportion of the new cells being produced which gave the indication that no new growth was occurring. We established replicate toxicity tests using the standard technique and the TFM concentrations of 15 and 30 mg/l, which exceeded the 96-h lethal concentrations for all species except Chlorella pyrenoidosa. After the cells had been exposed at these concentrations for 96 h, they were filtered free of the dosed medium and resuspended in TFM-free medium. This procedure simulated a natural situation in which an algal population is exposed to high lampricide concentrations for a relatively long period (4 days) in a bay or backwater at the mouth of a stream being treated for lamprey control. No growth was observed in any of the cultures during the initial 96-h exposure to 30 mg/l, and Chlorella pyrenoidosa was the only species to produce measurable growth at 15 mg/l of TFM.

Table 4. Maximum specific growth rates (μmax--average of three replicates) of 10 algal species at different concentrations of TFM

Species, and grade of TFM	TFM Concentration (mg/ℓ of active ingredient)						
	0	2.5	5.0	7.0	8.0	10.0	15.0
Scenedesmus quadricauda							
Analytical	1.230	0.867	0.815	0.351	0.231	0.239	0.201
Field	1.001	1.179	0.405	0.351	0.296	0.317	0.131
Stigeoclonium tenue							
Analytical	0.621	0.892	0.900	0.215	0.131	0.049	0.077
Field	0.718	0.476	0.270	0.166	0.140	0.049	0.000
Selenastrum capricornutum							
Analytical	1.751	1.825	1.959	1.839	1.546	1.405	1.497
Field	1.649	1.533	1.358	0.990	0.875	0.604	0.336
Chorella pyrenoidosa							
Analytical	1.619	1.445	1.351	1.440	1.834	1.488	1.660
Field	1.413	1.449	1.493	1.470	1.479	1.475	1.460
Cylindrospermum sp.							
Analytical	1.685	1.335	1.683	1.332	1.197	1.435	1.327
Field	1.224	1.264	1.163	0.486	0.836	0.767	0.692
Anabaena flos-aquae							
Analytical	0.784	0.900	0.667	0.582	0.412	0.095	0.093
Field	0.673	0.683	0.270	0.068	0.034	0.033	0.025
Nostoc linckia							
Analytical	0.811	0.747	0.723	0.658	0.593	0.631	0.525
Field	0.713	0.846	0.775	0.673	0.599	0.536	0.451
Anabaena cylindrica							
Analytical	1.212	1.241	0.728	0.315	0.515	0.195	0.221
Field	1.037	0.300	0.255	0.298	0.182	0.374	0.270
Nitzschia sp.							
Analytical	0.788	1.808	0.668	-0.930	-1.098	-	-
Field	1.037	0.104	0.104	-1.040	-1.099	-1.945	1.252
Navicula pelliculosa							
Analytical	1.229	1.253	1.184	0.693	0.095	-0.241	-0.105
Field	1.239	1.209	0.489	0.322	-0.134	0.255	0.104

Table 5. Maximum standing crop (dry weight in milligrams) of 10 species of algae after 96-h exposure to different TFM concentrations. Regression equations are given for species showing a linear response.

Species, and grade of TFM	TFM Concentration (mg/ℓ of active ingredient)							Equation
	0	2.5	5.0	7.0	8.0	10.0	15.0	
Scenedesmus quadricauda								
Analytical	8.8	7.0	4.4	0.4	0.6	0.4	0.0	$y = 7.495-0.650(X)$ $r^2=.779$
Field	4.6	6.8	2.0	0.0	2.0	0.0	0.0	
Stigeoclonium tenue								
Analytical	9.6	7.6	10.0	1.4	1.4	1.0	1.0	$y= 9.198-0.686(X)$ $r^2=.643$
Field	9.4	9.2	1.6	1.2	1.0	1.0	1.2	$y= 7.901-0.638(X)$ $r^2=.607$
Selenastrum capricornutum								
Analytical	4.0	4.2	3.8	2.8	1.6	1.2	0.0	$y = 4.694-0.388(X)$ $r^2=.830$
Field	3.8	3.0	1.8	0.2	0.0	1.2	0.0	$y = 3.948-0.473(X)$ $r^2=968$

Table 5. Maximum standing crop (dry weight in milligrams) of 10 species of algae after 96-h exposure to different TFM concentrations. Regression equations are given for species showing a linear response--Continued.

Species, and grade of TEM	TFM Concentration (mg/ℓ of active ingredient)							Equation
	0	2.5	5.0	7.0	8.0	10.0	15.0	
Chlorella pyrenoidosa								
Analytical	5.6	5.2	5.4	5.2	5.4	5.0	5.0	$y = 5.467-0.037(X)$ $r^2=.707$
Field	5.2	4.4	5.4	5.0	5.2	4.8	4.6	$y = 5.090-0.022(X)$ $r^2=.806$
Cylindrospermum sp.								
Analytical	12.0	8.0	9.2	6.2	5.4	2.4	5.2	$y = 10.324-0.503(X)$ $r^2=.635$
Field	5.4	6.8	5.0	3.6	4.0	1.6	0.8	$y = 6.535-0.391(X)$ $r^2=.834$
Anabaena flos-aquae								
Analytical	10.3	8.8	6.8	2.2	1.4	1.4	1.4	
Field	11.2	8.2	3.0	2.6	2.4	2.2	2.0	$y = 10.100-0.942(X)$ $r^2=.874$
Nostoc linckia								
Analytical	8.8	6.2	5.6	5.0	4.6	2.6	2.8	$y = 8.405-0.560(X)$ $r^2=.922$
Field	5.6	6.4	5.6	2.6	2.4	0.4	0.0	$y = 7.129-0.648(X)$ $r^2=.817$

Table 5. Maximum standing crop (dry weight in milligrams) of 10 species of algae after 96-h exposure to different TFM concentrations. Regression equations are given for species showing a linear response--Continued.

Species, and grade of TFM	TFM Concentration (mg/ℓ of active ingredient)							Equation
	0	2.5	5.0	7.0	8.0	10.0	15.0	
Anabaena cylindrica								
Analytical	3.0	1.8	0.4	0.0	0.0	0.0	0.0	$y = 2.809-0.393(X)$ $r^2=.951$
Field	3.8	0.0	0.0	0.0	0.0	0.0	0.0	
Nitzschia sp.								
Analytical	4.0	3.6	1.4	0.4	0.2	0.0	0.0	$y = 3.529-0.314(X)$ $r^2=.760$
Field	4.2	0.2	0.2	0.2	0.0	0.0	0.0	
Navicula pelliculosa								
Analytical	8.4	6.6	5.0	1.0	0.0	0.0	0.0	$y = 7.341-0.640(X)$ $r^2 = .780$
Field	6.8	3.8	0.0	0.0	0.0	0.0	0.0	

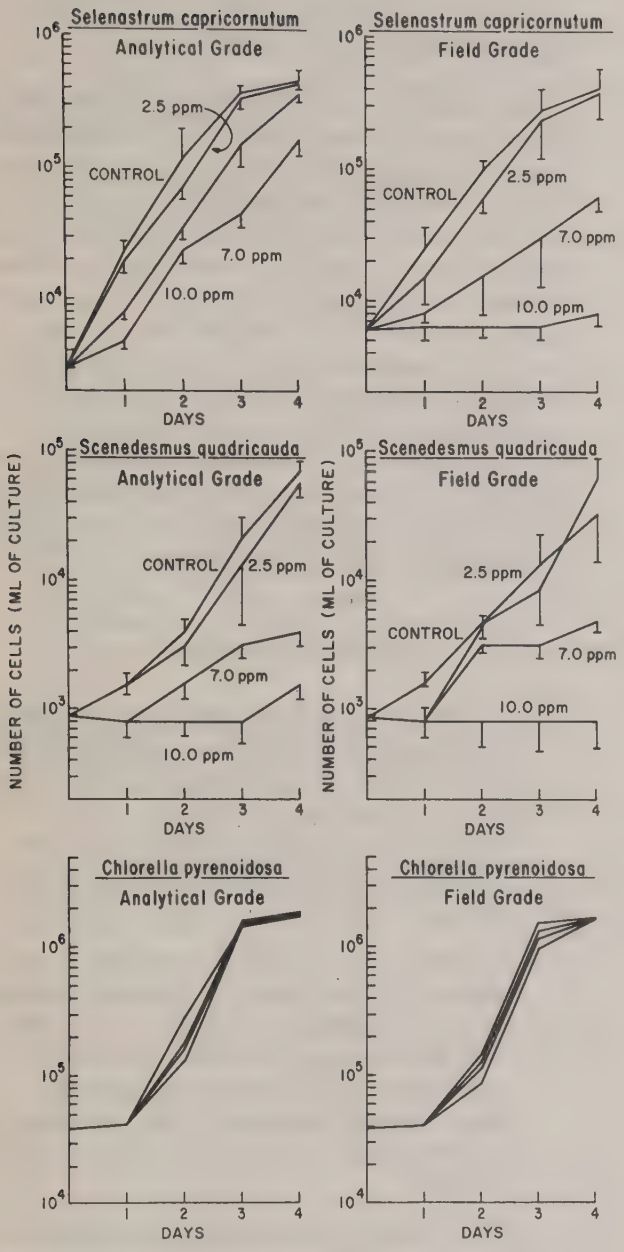

Fig. 3. Cell numbers in algal cultures grown in selected concentrations of analytical and field grade TFM.

After resuspension in toxicant-free medium, each alga showed little or no growth for 2 to 3 days. Soon after this lag, each culture grew, and cell density reached that in the controls after an average of 12 days, indicating that viability had not been destroyed at these high concentrations but that the ability to grow and reproduce was severely limited by the presence of the toxicant. The exception in this test was the sensitive diatom Navicula pelliculosa, which did not grow after the 96-h exposure to 30 mg/l of TFM.

The remaining possibility--that the cells were indeed growing in the high concentrations, but that the toxicant was selectively eliminating a proportion of the new cells being produced--remains unresolved. However, microscopic examinations of TFM-exposed algal cells while they were being counted during the toxicity tests tended to indicate that no vegetative reproduction was occurring, and that TFM was producing severe sublethal effects on the integrity of the chloroplast and other cell inclusions. Misshaped and shrunken chloroplasts were commonly observed in cells exposed to the higher concentrations of TFM, but normal growth and reproduction ensued soon after the cells were resuspended in toxicant-free medium.

Another possibility that warranted further investigation was that the color imparted to the algal growth medium by release of the phenolate ion from TFM selectively shades out a portion of the light spectrum and thus inhibits growth of the cultures. To test this hypothesis, we added sufficient food coloring to the growth medium to produce a yellow-green color that absorbed at 475 Klett Units--an absorption similar to that of a 30 mg/l solution of analytical grade TFM. Both species tested--Anabaena cylindrica and Selenastrum capricornutum--produced greater cell densities in 96 h in the flask to which food coloring had been added than in the control flasks. Although the absorption spectra of TFM and food coloring may not be exactly equivalent, we assumed on the basis of this test that shading from the colors of the phenolate ion of TFM did not limit growth.

DISCUSSION

Tests of the toxicity of analytical (95%) and field grade (35.7%) TFM to unialgal cultures of representative green and blue-green algae and diatoms showed that field grade material tended to exert the greater toxicity, when expressed on the basis of percentage active ingredient; 96-h 50% effect values generally were 2 to 3 mg/l lower for field grade than

for analytical grade material. Although the values were not statistically different, the tendency agrees with tendencies demonstrated in insects and fish.

The 96-h 50% effect levels ranged from 1.2 to more than 15 mg/l for field grade TFM and from 3.6 to more than 15 mg/l for analytical grade material. The most pronounced effects of the toxicant were observed in the diatom cultures, where 50% tolerance limits ranged from 1.2 to 5.2 mg/l. Intermediary effects were observed among the green algae, where toxicity values generally ranged from 4 to 8 mg/l; the exception was Chlorella pyrenoidosa which was resistant to concentrations greater than 15 mg/l. Morgan (1972) found that a unicellular green alga, Chlamydomonas reinhardtii, grew well at 20 mg/l of Aroclor 1242, a concentration normally lethal to other aquatic life. Zweig et al. (1968) found Chlorella pyrenoidosa to be relatively resistant to low concentrations of the herbicides diquat and 1,4-benzoquinone; the effects of the herbicides were termed algistatic rather than algicidal.

We found the more TFM-resistant species among the blue-green algae although two species of Anabaena had EC50's which approached those of the sensitive diatom species. This high susceptibility of Anabaena is unexplained.

Lampricide sensitivity differed little between species; i.e., in all of them, progressively lower growth rates were correlated with progressively higher toxicant levels. Several of the species expressed a higher μmax at 2.5 mg/l of TFM than did the controls, although the total biomass yield at the end of the 96-h test did not exceed the yield of control cultures. Stadnyk et al. (1971) reported similar increased growth rates and cell numbers, but not biomass, for cultures of Scenedesmus quadricauda exposed to 0.1 and 1.0 mg/l of organochlorine insecticides. The TFM may have been metabolized at low concentrations, either by the algal cells themselves or by contaminating bacteria. Although Klett Unit absorbance of filtered media did not change after any of the tests, the colorimeter may have been insufficiently sensitive to detect metabolism of the lampricide.

The present study indicates that residues of TFM inhibited algal growth at concentrations that might be developed during stream treatments. The inhibition was only temporary, however, and even exposures to the high concentration of 30 mg/l for 4 days did not decrease the viability of algal cells when they were resuspended in toxicant-free water. Although metabolic activity such as oxygen evolution or carbon assimilation were not measured, an effect on these activities is implied in the total reproductive capacity of the algal cultures. Further experimentation may yield an understanding of the nature of the temporary growth inhibition.

CONCLUSIONS

1. The lampricide TFM inhibited growth of all algal species tested. Concentrations of less than 10 mg/l caused a 50% inhibition of growth in all species except Chlorella pyrenoidosa, which was resistant to test concentrations up to 15 mg/l.
2. Diatoms were the most sensitive species tested; 50% inhibition of growth occurred at concentrations of 1-4 mg/l of TFM. The green algae were intermediate in sensitivity. In blue-green algae, the least susceptible group, 50% inhibition of growth generally occurred near 10 mg/l of TFM.
3. Field grade TFM generally tended to be more toxic than analytical grade material.
4. TFM does not appear to be algicidal within the range of concentrations tested but rather produces a temporary algistatic inhibition of growth.

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57. Acute Toxicities of 3-Trifluoromethyl-4-nitrophenol (TFM) and 2′,5-Dichloro-4′-nitrosalicylanilide (Bayer 73) to Larvae of the Midge Chironomus tentans

By Joseph A. Kawatski, Margaret M. Ledvina,
and Carl R. Hansen, Jr.

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ACUTE TOXICITIES OF 3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM) AND 2',5-DICHLORO-4'-NITROSALICYLANILIDE (BAYER 73) TO LARVAE OF THE MIDGE (CHIRONOMUS TENTANS)

By Joseph A. Kawatski, Margaret M. Ledvina, and Carl R. Hansen, Jr.
Department of Biology, Viterbo College
La Crosse, Wisconsin

ABSTRACT

The toxicants 3-trifluoromethyl-4-nitrophenol (TFM) and 2',5-dichloro-4'-nitrosalicylanilide (Bayer 73) were tested individually and together for toxicity to fourth instar Chironomus tentans in laboratory static tests at 22 $\pm$ 1 C. The 24-h EC50 (immobility) values in soft water (total hardness, 40-48 mg/ℓ as $CaCO_3$) were 1.55 mg/ℓ of 95.7% TFM, 0.570 mg/ℓ of 70% Bayer 73, and 0.658 mg/ℓ of total toxicant for a 98:2 mixture of TFM and Bayer 73. As water hardness increased, toxicity of the materials individually and in mixtures decreased. Generally, the toxic effect of mixtures of TFM and Bayer 73 was additive; synergistic toxicity was apparent only in soft water at exposures of 24 to 72 h. Immobility was not coincident with death in chironomids. Considerably greater concentrations of toxicant were required to kill than to immobilize.

INTRODUCTION

The toxicant 3-trifluoromethyl-4-nitrophenol (TFM) has been used extensively and successfully to control the sea lamprey (Petromyzon marinus) in the Great Lakes. Authorization for the continued use of the chemical will depend on the outcome of current research designed to clarify the effects of TFM on a variety of nontarget organisms (Schnick 1972). The molluscicide Bayer 73 (2-aminoethanol salt of 2',5-dichloro-4-nitrosalicylanilide) is applied with TFM to produce a 2% mixture, i.e., 98 parts of TFM (95.7%): 2 parts Bayer 73 (70% W.P.), here termed TFM:2B. This mixture has an apparent synergistic effect on lamprey larvae (Howell et al. 1964). Inasmuch as invertebrates constitute a vital portion of aquatic food webs, the elucidation of the toxicities of TFM and Bayer 73 to invertebrates is essential.

Among the invertebrates in most aquatic food chains, insect larvae, including midges, represent one of the most important links at a relatively low trophic level. We report here on the acute toxicities of TFM, Bayer 73, and TFM-2B to laboratory-reared, fourth instar populations of Chironomus tentans Fabricius, a widely distributed benthic midge and an important fish food organism. For reference, we also determined the toxicity of antimycin A against this chironomid.

MATERIALS AND METHODS

The U.S. Fish and Wildlife Service, Fish Control Laboratory, La Crosse, Wisconsin, provided the following materials for this study: TFM (95.7% 3-trifluoromethyl-4-nitrophenol, Aldrich Chemical Co., Lot No. 060217); Bayer 73 (70% 2-aminoethanol salt of 2',5-dichloro-4'-nitrosalicylanilide, wettable powder, Chemagro Corp., Lot No. 8059410); antimycin A (95.5%, Ayerst Laboratories, Lot No. 1294-L); and reconstituted water. Soft, hard, and very hard waters, with respective total hardnesses of 40-48, 160-180, and 280-320 (as mg/ℓ $CaCO_3$), were used in the toxicity tests. These waters were further characterized by Marking (1970). The food and substrate mixture consisted of ground Trainers Dog Rewards® (Horlick's Corp., Racine,

Wisconsin) and macerated paper hand towels (Crown Zellerback Corp., San Francisco, California). This mixture contained no detectable TFM or Bayer 73 residues as determined by gas-liquid chromatographic analysis.

Stock cultures of C. tentans were propagated in the laboratory in 5-, 20-, and 400-liter glass or fiberglass aquaria, in soft, reconstituted water at a temperature of 22 ± 1.5 C, under a 16-h photoperiod of mixed fluorescent and incandescent light. Food and substrate were added periodically to sustain growth and reproduction. Continuous gentle aeration was provided. This rearing system was essentially that of Derr and Zabik (1972).

About 48 h before each toxicity test, we transferred 10-20 organisms individually, with a curved blunt probe, into separate beakers containing 900 mℓ of well-aerated test water. The toxicants, dissolved in 50% acetone (in water), were then introduced into the exposure vessels to produce the appropriate test concentrations. The total amount of acetone added to each vessel and to control systems was 0.5 mℓ. The criterion for evaluating toxicant effect was immobility, noted after 8, 24, 48, 72, and 96 h of exposure. A test animal was considered immobile when it failed to move its body, jaws, or anal gills after it was touched lightly with a glass probe. The water was not aerated during exposures. Toxicity tests with TFM, Bayer 73, and TFM-2B were replicated at least three times for each of three water hardnesses at 22 ± 1 C. Using the Litchfield and Wilcoxon (1949) method, we calculated the EC50's from the combined data of at least three independent tests. (The EC50 is defined as the concentration of toxicant, based on total formulation weight, that immobilized 50% of the test organisms within the prescribed period of exposure.) Results of tests in which more than 20% of the control organisms died were discarded. From 114 to 590 animals were used to determine the EC50's for each chemical or combination of chemicals. The EC50's derived for TFM-2B were based on total toxicant, i.e., the sum of TFM and Bayer 73.

The toxicity of TFM-2B was compared with the toxicities of the two lampricides alone by calculation of additive indices as described by L. L. Marking and V. K. Dawson (in preparation). The significance of index values is determined from ranges for the indices, using 95% confidence limits for the EC50 values. If the range for an index is above zero, the toxicity of the combined chemicals is synergistic; if the range is below zero, the toxicity is antagonistic.

RESULTS

Water quality had a direct effect on the toxicity of TFM, Bayer 73, and TFM-2B against C. tentans (Table 1.) Hard water with high pH (8.0-8.4) decreased the toxicity of the chemicals, whereas soft water with a lower pH (7.2-7.6) increased it. The influence of water quality was less pronounced with Bayer 73 than with TFM. In comparison with TFM, the toxicity of Bayer 73 to C. tentans was 2 to 3 times greater in soft water and 6 to 12 times greater in hard water.

At most water hardnesses and exposure periods, the addition of 2% of Bayer 73 reduced the amount of TFM required to immobilize chironomids. However, the ranges of additive indices for TFM-2B indicated that synergistic toxicity occurred only in soft water and then only at three intermediate exposure periods (Table 2). Additive index ranges overlapped zero in all water hardnesses at 8- and 96-h exposures; when test water was hard or very hard, the toxic effect of TFM-2B was additive.

Antimycin A was used as a reference chemical to determine the sensitivity of our laboratory population of C. tentans. In soft water, the 96-h LC50 and 95% confidence limits were determined to be 0.146 (0.095-0.224) μg/ℓ.

DISCUSSION

Howell et al. (1964), who determined the lethal concentrations of TFM to lamprey larvae in water ranging from soft with a pH of 7.3 to very hard with a pH 8.5, reported that toxicity decreased as hardness and pH increased. They found, however, that even in hard water the amount of TFM-2B required to

Table 1. Toxicities of TFM, Bayer 73, and a 98:2 mixture of TFM and Bayer 73 to fourth instar Chironomus tentans in waters of different ranges of hardness at 22 ± 1 C

Chemical and water hardness (mg/ℓ as $CaCO_3$)	EC50 (mg/ℓ)[a] and 95% confidence intervals at:				
	8 h	24 h	48 h	72 h	96 h
TFM					
Soft (40-48)	1.65 1.45-1.88	1.55 1.32-1.83	0.968 0.815-1.15	0.790 0.631-0.989	0.534 0.415-0.688
Hard (160-180)	6.47 5.66-7.39	4.54 4.06-5.08	2.53 2.13-3.01	1.19 0.937-1.51	0.998 0.854-1.17
Very hard (280-320)	14.3 13.1-15.6	10.0 8.09-12.4	6.60 5.47-7.97	3.49 2.84-4.29	2.10 1.65-2.67
Bayer 73					
Soft (40-48)	0.744 0.550-0.998	0.570 0.458-0.710	0.367 0.289-0.467	0.347 0.257-0.469	0.228 0.171-0.304
Hard (160-180)	1.01 0.712-1.43	0.640 0.501-0.817	0.457 0.352-0.593	0.360 0.275-0.471	0.295 0.221-0.393
Very hard (280-320)	2.01 1.45-2.79	0.814 0.630-1.05	0.536 0.417-0.690	0.374 0.286-0.490	0.353 0.261-0.477
Mixture, TFM, and Bayer 73 (98:2)					
Soft (40-48)	1.38 1.18-1.61	0.658 0.474-0.913	0.400 0.312-0.513	0.358 0.283-0.453	0.328 0.262-0.416
Hard (160-180)	4.33 3.76-4.99	2.59 1.76-3.81	1.52 1.19-1.95	0.910 0.749-1.11	0.657 0.516-0.821
Very hard (280-320)	11.8 10.7-13.1	7.03 5.89-8.39	4.94 4.14-5.89	3.50 2.81-4.36	2.79 2.36-3.29

[a] EC50 (immobility) values for mixture are expressed as total toxicant (TFM plus Bayer 73).

Table 2. Additive indices for the toxicity of a 98:2 mixture of TFM and Bayer 73 against Chironomus tentans in selected harndesses at 22 ± 1 C

Water hardness (as mg/ℓ $CaCo_3$)	Additive indices[a] and their ranges at:				
	8 h	24 h	48 h	72 h	96 h
Soft (40-48)	+0.167 -0.147-+0.566	+1.28 +0.930-+2.74	+1.34 +0.533-+2.58	+1.15 +0.354-+2.42	+0.585 -0.031-+1.56
Hard (160-180)	+0.348 -0.004-+0.814	+0.562 -0.072-+1.61	+0.526 -0.008-+1.34	+0.250 -0.242-+0.931	+0.450 -0.016-+1.18
Very hard (280-320)	+0.080 -0.161-+0.335	+0.161 -0.283-+0.731	+0.900 -0.338-+0.590	-0.170 -0.809-+0.322	-0.460 -1.21-+0.036

[a] Indices with ranges less than zero suggest antagonism; indices with ranges that overlap zero suggest additive toxicity; and indices with ranges, greater than zero suggest synergism.

kill lampreys was less than the amount of TFM alone required to kill them. B. R. Smith (1967) also observed that the toxicity decreased as water hardness increased when Bayer 73 and TFM-2B were tested against lampreys and rainbow trout (Salmo gairdneri), and that the degree of synergism decreased as water hardness and pH increased. Our data and those of Kawatski (1973) show that the response of midges and ostracods is similar to that of fish.

Some chironomids that were immobilized by TFM and appeared dead revived when they were placed in toxicant-free water. Post-exposure observation enabled us to establish that, for TFM in soft water, the 8-h LC50 was 12 times greater than the immobility-based 8-h EC50. The disparity between EC50 and LC50 values resides with the difficulty in ascertaining whether a test animal is immobile, moribund, or dead. However, as exposure time lengthens, dead chironomids are more easily recognized, and the difference between the EC50's and LC50's in longer exposures (24-96 h) is much smaller.

Recovery of test organisms, especially after short-term exposure, was not surprising because earlier experiments (unpublished) in our laboratory showed that chironomids exposed to ^{14}C-TFM rapidly biotransformed and excreted accumulated residue; this detoxication continued even after the organisms were immboilized. Because of these observations and the difficulty in defining death, particularly in small and/or normally sluggish invertebrates, it is clear to us that in short-term exposures LC50's can be accurately determined only if the affected organisms are held in toxicant-free water after the exposure period.

CONCLUSIONS

1. The toxicity of TFM, Bayer 73, and TFM-2B against Chironomus tentans decreases as water hardness and pH increase.

2. Generally, the toxic effect of TFM and Bayer 73 in combination (TFM-2B) is additive; synergistic toxicity occurs only in soft water at exposure times of 24 to 72 hours.

3. Toxicant-induced immobility was not synonymous with death, as shown by the large percentage of toxicant-immobilized larvae that became active when they were placed in TFM-free water after exposure.

ACKNOWLEDGMENT

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58. Acute Toxicity of the Lampricide 3-Trifluoromethyl-4-nitrophenol (TFM) to Nymphs of Mayflies (Hexagenia sp.)

By Calvin R. Fremling

United States Department of the Interior
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ACUTE TOXICITY OF THE LAMPRICIDE 3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM) TO NYMPHS OF MAYFLIES (HEXAGENIA SP.)

By Calvin R. Fremling
Winona State College
Winona, Minnesota

ABSTRACT

A recycling test apparatus and burrow-containing artificial substrates were used to determine the toxicity of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) against Hexagenia mayfly nymphs. Toxicity was relatively independent of temperature, but was greater in soft water than in hard water, and much greater at low than at high pH's; 12-h LC50's were 4.0 at pH 6.5 and 270.0 at pH 9.5.

INTRODUCTION

Hexagenia mayflies play a vital ecological role in lakes, rivers, and streams (Needham et al. 1935, Hunt 1953, Britt 1955, Fremling 1960, Swanson 1967). Nymphs of Hexagenia are important to fish because they convert organic detritus, algae, and bacteria into high quality fish food. The detritus-mayfly-fish food chain is short and efficient. Inasmuch as the life cycle of Hexagenia lasts at least a year in the Great Lakes and in most tributary streams, nymphs are available to fish in all seasons. Because the nymphs pass through many molts, sizes are available to suit the needs of most fish species.

Since Hexagenia nymphs prefer silt bottoms where they can construct burrows, they usually inhabit the same areas used by lamprey ammocoetes. Silted streams, for example, provide suitable habitat for both. It is important to determine the effect of lampricides on Hexagenia nymphs because they may be eradicated in lamprey control areas if the lampricides are toxic to them.

Hexagenia nymphs are good test organisms because they are easily collected and cultured and their large size makes them easy to handle and observe. Their tendency to abandon their burrows when stressed makes it possible to accurately assess early effects of toxicants.

METHODS

Either reconstituted water supplied by the Fish Control Laboratory, La Crosse, Wis., or water from a 12-m deep sand point well was used in the tests. The reconstituted water contained 48 mg/l of $NaHCO_3$, 30 mg/l of $CaSO_4$, 30 mg/l of $MgSO_4$, and 2 mg/l of KCl; was slightly alkaline (pH 7.2-7.6); and was soft (hardness 40-48 and alkalinity 30-35 as mg/l of $CaCO_3$).

The well water was hard, having a total alkalinity of 331, total hardness of 384, and calcium hardness of 260 (all as mg/l of $CaCO_3$). Resistivity at 25 C was 1,277 ohms and pH was 7.42. Chemical constitutents (mg/l) included ammonia nitrogen, 0.38; nitrite, 0.005; nitrate, 0.05; sulfate, 42.5; orthophosphate, 0.05; total iron, 0.28; manganese, <0.05; sodium, 32.5; calcium, 59.0; magnesium, 18.3; and potassium, 3.7.

Hexagenia nymphs were used as experimental animals, and cultures were maintained in the laboratory according to methods described by Fremling (1967). Relatively large nymphs (20-22 mm) were used in all experiments because they were easy to handle and observe. Last instar nymphs were not knowingly used because physiological stresses involved in transformation to the adult stage are atypical and emergence during the experiments was not desired. Although most test nymphs were H. bilineata from laboratory

cultures, their number was supplemented in all experiments by nymphs of H. bilineata and H. limbata collected from the Mississippi River. Species collected from the river were not separated because the nymphs were not in their last instar and because undue handling was undesirable. Nymphs collected from the river were placed in laboratory rearing tanks to acclimate for at least 1 wk before they were used in tests.

Nymphs were collected from the rearing tanks by gently sifting mud through a coarse screen; they were then transferred to fresh water where they acclimated for 6 h before being used in tests. A large syringe, filled with test water from the appropriate vessel, was used to flush the remaining nymphs from their burrows to determine if any were dead. Each nymph was classified as normal, dead, or stressed (as indicated by active swimming, rapid gill movements, or loss of equilibrium). All tests were conducted in a basement laboratory which had no windows. Overhead incandescent lamps provided constant light.

Special glass toxicity test vessels (Fig. 1) were assembled with silicone glue. Each

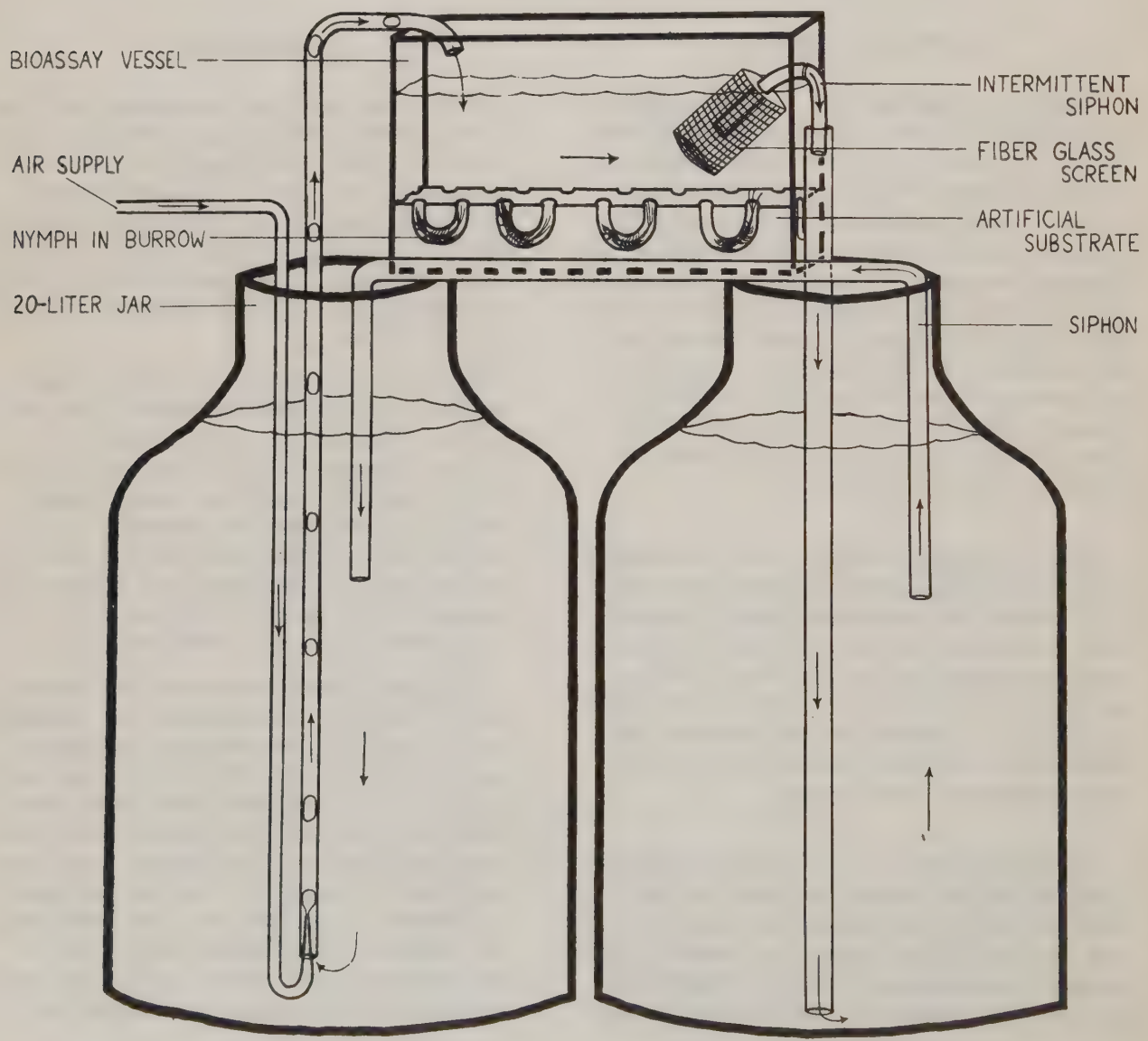

Fig. 1. Recycling bioassay apparatus with a burrow-containing epoxy substrate for use with Hexagenia mayfly nymphs and nonvolatile test chemicals.

vessel contained an epoxy resin substrate, 23 cm long, 5 cm wide, and 5 cm deep. Each substrate contained 10 burrows constructed as described by Fremling and Schoening (1973). Constant recirculation of test water through each vessel was assured by pumping water via an air lift (made of 4 and 6 mm I.D. glass tubing) from a 20-liter glass jar through the test vessel to another 20-liter glass jar via an intermittent siphon. A fiber glass screen prevented the escape of test animals. Water was constantly returned by a siphon (10 mm I.D. glass tubing) from the second jar to the first. Recirculation of the test water, which was permissible because TFM is relatively nonvolatile, made it possible to maintain precise control over toxicant concentrations. Accumulation of inhibitory concentrations of degradation and metabolic products was unlikely because of the large volume of water used (38 liters).

Six two-jar units were placed in each of two large water baths so that temperature could be accurately controlled. Each series of six units included one control unit in which no TFM was used and five units which contained various concentrations of TFM.

Stock solutions of field grade TFM (35.7%) and purified TFM (95%) were made by dissolving the chemical in acetone and diluting it with water. Stock solution was added in equal amounts to both jars of each unit, stirred thoroughly, and allowed to circulate between the jars until mixing was complete. Concentrations of TFM were monitored before nymphs were added and periodically throughout each experiment with a Beckman DB spectrophotometer (Olson and Marking 1973).

Waters of various hardnesses and pH's were used, as described by Marking (1969) and Marking and Dawson (1972), respectively. LC50 values and 95% confidence limits for each test were determined according to methods described by Litchfield and Wilcoxon (1949).

RESULTS

Purified and field grade TFM were toxic to mayfly nymphs at all temperatures tested (17.0 - 26.5 C--see Table 1). The change in toxicity was usually insignificant ($P>0.05$) for single temperature increments; an exception being the increment between 23.5 and 24.4 C in hard water at 24-h exposure. The exception indicates that TFM is more toxic at the higher of the two temperatures. Since the temperature difference is small, however, the data may reflect biological variation among groups of organisms rather than an influence of temperature. Although TFM is toxic in short

Table 1. Toxicity of TFM (based on active ingredient) to Hexagenia mayfly nymphs in waters of different hardness and temperature. LC50 values and 95% confidence intervals (in parentheses) are listed as µl/l for 35.7% TFM and as mg/l for 95% TFM.

Hardness (mg/l)	Temp. (°C)	Formulation of TFM (%)	Hours of exposure			
			6	12	24	96
384	17	35.7	-- --	10.5 (8.47-13.0)	6.50 (5.23-8.07)	3.90 (3.0-5.07)
384	21.8	95	-- --	-- --	6.00 (5.18-6.95)	4.30 (3.45-5.36)
384	23.5	95	--	10.5 (9.38-11.8)	7.00 (5.79-8.47)	4.20 (3.41-5.17)
384	24.4	95	11.2 (10.0-12.5)	6.50 (5.21-8.11)	3.50 (2.28-5.36)	-- --
44	18.2	35.7	-- --	-- --	4.75 (4.20-5.37)	2.50 (1.80-3.46)
44	26.5	35.7	-- --	4.70 (3.96-5.58)	3.50 (2.96-4.14)	2.18 (1.73-2.74)

exposures (12-h or less), LC50's at these exposures are not much greater than those for 24-h exposures. Comparisons made over a wider temperature range might show greater significance.

Water hardness influenced the toxicity of TFM to Hexagenia nymphs. After 24-h exposures, TFM was considerably more toxic in soft water than in hard water of similar pH (7.1-7.6) and temperature (Table 1). The 24-h LC50's were 6.50 and 4.75 µl/l of TFM in hard and soft water, respectively, at the lower temperatures (17.0 and 18.2 C). At the higher temperatures (24.4 and 26.5 C), TFM was more toxic in soft than in hard water at the 12-h exposure but the difference was nil at 24-h.

The toxicity of TFM to Hexagenia mayfly nymphs was influenced drastically by the pH of water (Table 2). The 24-h LC50 at pH 6.5 (2.50) was significantly greater ($P<0.05$) than that at pH 7.5 (3.35), and the LC50 at pH 8.5 (18.8) was more than 5 times the value at pH 7.5 (3.35). The toxicity of TFM to the nymphs was lowest at pH 9.5 and the 24-h LC50 was almost 70 times greater than that value at pH 6.5.

DISCUSSION

TFM is apparently less toxic to some invertebrate animals than it is to lampreys. Experiments by Erkkila (1962) revealed that concentrations of TFM below 20 µl/l caused insignificant mortality to isopods, gammarids, crayfish, dragonflies, water boatmen, and case-building caddisflies; that concentrations below 10 µl/l were harmless to glossiphonid leeches, stoneflies, bloodworms and snails; and that mortality was significant in Hexagenia at 6 µl/l. Smith (1967) showed that mortality of hydras, turbellarians, blackflies, and Hexagenia mayflies was almost complete in the laboratory when these animals were exposed to TFM at 10 µl/l for prolonged periods.

In the present study, TFM was toxic to Hexagenia mayflies in ranges similar to those reported above. Except at pH of 8.5 or over, the 24-h LC50 of TFM was always less than 10 µl/l. At low pH in soft water the material was especially toxic. At pH 6.5, for example, the 96-h LC50 was 1.18 (Table 2).

Table 2. Toxicity of TFM (35.7%) to Hexagenia mayfly nymphs in soft water (40-48 mg/l total hardness as $CaCO_3$) at temperatures of 22-23 C and at selected pH values. LC50 values and 95% confidence intervals (in parentheses) are listed as µl/l TFM.

pH	Hours of exposure				
	12	24	48	72	96
6.5	4.00 (3.47-4.61)	2.50 (2.16-2.90)	1.31 (1.04-1.65)	-- --	1.18 (0.91-1.53)
7.5	-- --	3.35 (2.99-3.76)	2.50 (2.17-2.87)	2.00 (1.63-2.46)	-- --
8.5	27.3 (22.4-33.0)	18.8 (16.6-21.4)	13.0[1]	-- --	5.00 (3.68-6.80)
9.5	270 (221-329)	174 (156-194)	100 (87.2-115)	64.2 (49.5-83.3)	60.0 (45.7-78.8)

[1]No confidence interval reported because of insufficient data.

Hexagenia nymphs are less sensitive than larval lampreys to TFM when both species are tested in standard laboratory water. Dawson et al. (in press) found the 24-h LC99 for ammocoetes to be 0.90 at pH 6.5, 3.25 at pH 7.5, and 12.0 at pH 8.5. In the present study, 24-h LC50 values for Hexagenia nymphs were 2.50 at pH 6.5, 3.35 at pH 7.5, and 18.8 at pH 8.5.

In all tests there was a marked tendency for treated nymphs to abandon their burrows for varying lengths of time before they actually succumbed. In nature, this behavior would, on one hand, increase the vulnerability of nymphs to predation; on the other hand, however, free-swimming nymphs might swim, or be swept by the current, out of the zone of lethal TFM concentrations. There is no assurance, however, that nymphs would find suitable substrate in an open lake or that they would recover from the effects of the toxicant.

Hexagenia mayflies are able to recolonize denuded areas by downstream drift of nymphs and by upstream flight of ovipositing adults (Fremling 1973). It is likely that Hexagenia populations killed by TFM applications would become reestablished. Complete reestablishment would probably require a year or more, however.

The artificial substrate apparatus used in this study proved very satisfactory as indicated by the fact that in 10 96-h tests the controls showed no mortality in three tests, 10% in five tests, 20% in one test, and 30% in one test. In the test in which mortality was 30%, two of the dead were nymphs which died during transformation to the subimaginal stage. Nymphs frequently molted to the next nymphal instar in the bioassay vessels.

Artificial substrates such as those used in this study provide semidarkness, thigmotactic surfaces, and seclusion for test nymphs. Consequently, the nymphs swim less and their susceptibility to toxicants is not enhanced by fatigue as it is in standard test vessels.

CONCLUSIONS

1. A recycling toxicity test apparatus with artificial substrates was suitable for tests of TFM against Hexagenia nymphs.

2. Toxicity of TFM to Hexagenia nymphs is relatively independent of temperature.

3. Toxicity of TFM to Hexagenia nymphs is greater in soft than in hard water.

4. Toxicity of TFM to Hexagenia nymphs is much greater at low than at high pH's.

5. Although TFM is more toxic to ammocetes than to Hexagenia nymphs in soft water, applications of TFM that exceed the minimum effective concentrations for lamprey larvae may kill the nymphs.

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INVESTIGATIONS IN FISH CONTROL

59. Toxicity and Residue Dynamics of the Lampricide 3-Trifluoromethyl-4-nitrophenol (TFM) in Aquatic Invertebrates

By Herman O. Sanders and David F. Walsh

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TOXICITY AND RESIDUE DYNAMICS OF THE LAMPRICIDE 3-TRIFLUOROMETHYL-4-NITROPHENOL (TFM) IN AQUATIC INVERTEBRATES

By Herman O. Sanders and David F. Walsh
Fish-Pesticide Research Laboratory, Columbia, Missouri

ABSTRACT

Six species of aquatic invertebrates including scud, Gammarus pseudolimnaeus, daphnid, Daphnia magna, crayfish, Orconectes nais, aquatic sowbug, Asellus brevicaudus, damselfly nymph, Ischnura verticalis, and a mayfly nymph, Stenonema sp. were exposed to TFM in toxicity tests in hard water at 21 C.

The 96-h LC50 of field grade TFM (35.7%) was 57.0 mg/l for scud and 110 mg/l for crayfish; purified TFM (95.7%) was twice as toxic. The thirty-day LC50 of field grade TFM was 14 mg/l for scud and 20 mg/l for crayfish. LC50 values are based on whole formulation rather than active ingredient.

Uniformly ^{14}C-ring labeled TFM was employed in the accumulation experiments. All organisms accumulated TFM concentrations within 7 days that were up to 58 times (wet weight of whole organism) the concentration in water. The biological half-life of TFM in scud was 3.5 days. No reproductive impairment occurred in daphnids exposed to 2.4, 4.9, and 10 mg/l field grade TFM for three generations (63 days). Reproduction stopped, however, within the first generation when exposed to 18 mg/l.

INTRODUCTION

The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) was registered in 1964 for limited use in tributaries of the Great Lakes for control of the parasitic sea lamprey (Petromyzon marinus). In 1970, however, the registration of TFM was cancelled by the Environmental Protection Agency because of insufficient information on its residues and impact on aquatic organisms other than lamprey. Since cancellation, extensions have been granted to provide time for the Great Lakes Fishery Commission to generate the necessary information for registration.

The physical and chemical properties, efficacy, and toxicity of TFM to aquatic organisms have been compiled in an excellent review by Schnick (1972). This review is extensive and indicates that only a few studies report on the toxicology of TFM in aquatic invertebrates. Laboratory and field studies have shown that TFM is not acutely toxic to most fish (Applegate and King 1962; Applegate et al. 1961) and invertebrates (Erkkila 1962; Smith 1967) when used at concentrations that kill lamprey larvae (Applegate et al. 1958).

Laboratory studies with TFM and invertebrates have been concerned primarily with acute toxicity. Since accumulation and biological effects of TFM residues in aquatic invertebrates are not understood, we initiated this study to determine toxicity, uptake, dissipation, and residue magnification of TFM in invertebrates. In addition, we evaluated the effects of TFM on reproduction in daphnids, Daphnia magna. The data in this report are intended to assist in the registration of TFM and in establishing criteria for permissible

TFM concentrations in the aquatic environment.

MATERIALS AND METHODS

Test Animals

Test animals consisted of four species of crustaceans and two species of early instar aquatic insects: mature scud (Gammarus pseudolimnaeus Bousfield); early instar and mature daphnids (Daphnia magna Strauss); 14-day-old crayfish (Orconectes nais Faxon); mature aquatic sowbug (Asellus brevicaudus Forbes); damselfly nymph (Ischnura verticalis Say); and mayfly nymph (Stenonema sp.). Daphnids were from laboratory cultures; all other invertebrates were collected from streams and ponds near the Fish-Pesticide Research Laboratory, Columbia, Missouri. Invertebrates collected in the field were placed in acclimation tanks for at least 7 days before testing.

The water used for cultures and all experiments was from a deep well and had the following characteristics: pH 7.2-7.4 and total hardness 270 mg/l as $CaCO_3$.

Toxicity Tests

The acute toxicity of field grade TFM (35.7% active ingredient) and purified TFM (95.7% active ingredient) was determined by the standard 96-h static toxicity test (Sanders 1970) and LC50 values were calculated on the basis of the total formulation. Thirty-day flow-through tests with field-grade TFM were conducted using a flow-through diluter after Mount and Brungs (1967). In the flow-through tests, scud were fed coarsely chopped maple and elm leaves and crayfish were fed enriched fish-food pellets. All tests were conducted at 21 ± 1°C.

Toxic effects were measured in terms of the median lethal concentration (LC50), the toxicant concentration in water which is lethal to 50% of the test animals under the test conditions. In flow-through tests, the incipient LC50 or lethal threshold concentration (Sprague 1969) was determined when the asymptote had been reached in the toxicity curve. This value was determined when the mortality in each aquarium in any 5-day period dropped to 10% of the original number of animals. Toxicity estimates (LC50 values) and corresponding 95% confidence intervals were determined by the Litchfield Wilcoxon method (1949).

Uptake Method

Uptake of TFM from water by the four species of crustaceans and two species of immature insects was studied at concentrations of 0.013, 0.020, 0.026, and 0.510 mg/l.

Uniformly ^{14}C-ring labeled TFM (specific activity 3.66 mCi/mM) was used in the accumulation experiments. A sample of TFM examined by direct probe mass spectrometry contained 0.02% non-volatile ^{14}C-impurities, but no impurities of higher molecular weight were observed (Analyst, D. L. Stalling, Fish-Pesticide Research Laboratory, Columbia, Mo.).

Stock solutions of ^{14}C-TFM were prepared in water and further diluted to desired concentrations in a flow-through system. The water in each aquarium was renewed at a rate of 120 ml/h. The organisms were exposed in two-liter glass aquaria containing one liter of well water. The flow-through system was operated for at least 24 h prior to addition of organisms to allow for concentration equilibrium. The organisms were not fed during the accumulation experiments.

Invertebrate samples were taken in triplicate, weighed, and prepared directly for radiometric analyses by homogenizing the whole organism in a tissue grinder. The homogenate was obtained by adding 6 ml of Triton X-100®: toluene (2:3 v/v) emulsifier to each sample during grinding (Johnson et al. 1971). This homogenate was then transferred to a scintillation vial with three 3 ml washings of a toluene-fluor mixture (5 g of diphenyloxazole (PPO) in 1 liter of toluene). The concentration of TFM in water was monitored radiometrically by taking triplicate 1 ml samples of aquarium water directly into a scintillation vial and then adding 14 ml of Triton/toluene-fluor mixture. The radioactivity

in the tissue and water samples was measured with a Beckman 200-L liquid scintillation spectrometer. Residue values and magnification factors (residue concentration in organism/residue concentration in exposure water) presented in the text and tables were computed on a whole-body, wet weight basis.

Dissipation Method

Dissipation of TFM residues in scud was determined by exposing the organisms to TFM until a plateau concentration was reached. The scud were then transferred to TFM-free flowing water and analyzed periodically to measure decline in whole-body residues.

Reproduction Studies

Reproductive studies with daphnids were conducted in a flow-through system designed for exposing small organisms to constant concentrations of a toxicant over an extended period. Ten first-instar daphnids, up to 24 h old, were placed in duplicate exposure vessels containing 1 liter of water. An aqueous stock solution of field grade TFM was prepared and then further diluted with water to concentrations of 2.4, 4.9, 10, and 18 mg/l. A control was included with each test. Daphnids were fed a suspension of yeast and algae in sufficient amounts to support a stable population. Reproductive success was assessed by counting the offspring produced in each TFM concentration after the parent daphnids had been exposed for 21 days. At the end of 21 days, 10 of the young from each concentration were placed in new media and the 21-day procedure was repeated.

RESULTS

Acute toxicity

Static 96-h toxicity tests indicated that field grade TFM (35.7% AI) and purified TFM (95.7% AI) have relatively low acute toxicities to scud and crayfish (Table 1). The 96-h LC50 value of field grade TFM was 57 mg/l for scud and 110 mg/l for crayfish. The 96-h LC50 value of purified TFM was 22 mg/l for scud and 55 mg/l for crayfish. The toxicity to both animals appears to be related to the level of active ingredient of the compound.

Chronic toxicity

Thirty-day flow-through toxicity tests of field grade TFM with scud resulted in progressively lower LC50 values ranging from 43 mg/l at 5 days to 14 mg/l at 30 days (Table 2). The incipient LC50 was 14 mg/l which was attained in 20 days. The LC50 values for crayfish exposed to field grade TFM declined from greater than 100 mg/l at 1 day to 20 mg/l at 30 days. The incipient LC50 for crayfish was 20 mg/l.

The LC50 values for 4-day exposures of scud to field grade TFM were similar in static and flow-through tests. However, crayfish exposed for 4 days in the flow-through tests were resistant to TFM concentrations twice as high as those in the static test.

Uptake Study

All invertebrates exposed continuously to sublethal concentrations of ^{14}C-TFM accumulated radioactive residues in 7 days that were up to 58 times the concentration in water (Table 3). After an initial rapid uptake, most invertebrates accumulated TFM at a slow rate until a plateau was reached at 7 days. Daphnids, however, accumulated plateau concentrations after a 1-day exposure.

Accumulation of TFM residues by invertebrates appears dependent upon the concentration in water, but magnification factors are relatively independent of these concentrations. After a 7-day exposure to 0.013 mg/l, scud concentrated TFM 58 times (0.754 mg/kg) the level in water. When scud were exposed to 0.510 mg/l, they accumulated total body concentrations 56 times (28.6 mg/kg) that of water.

A comparison of the results from our accumulation experiments indicates a significant difference in the rate of uptake and residue magnification of TFM by the various organisms. TFM uptake from water by aquatic insects was relatively low when compared to uptake by crustacea. However, of all the organisms investigated, crayfish accumulated the least TFM concentrations from water.

Table 1. Toxicity[1] of field grade TFM and purified TFM to scud and crayfish

Organism	LC50 values[2] (mg/l) and 95% confidence intervals at --			
	24 h		96 h	
	Field grade	Purified	Field grade	Purified
Scud (mature)				
Gammarus pseudolimnaeus..	100 (83-130)	28 (23-34)	57 (47-69)	22 (16-31)
Crayfish (14 days old)				
Orconectes nais.....	130 (115-150)	60 (45-80)	110 (90-125)	55 (48-70)

[1]Static toxicity test. Hard water (pH, 7.2-7.4, total hardness 270 mg/l as $CaCO_3$ at 21° C.
[2]Values based on whole formulation rather than active ingredient.

Table 2. Flow-through toxicity tests[1] of field grade TFM against scud and crayfish

Organism	LC50 values[2] (mg/l) and 95% confidence intervals at --					
	1 day	4 days	10 days	15 days	20 days	30 days
Scud (mature)						
Gammarus pseudolimnaeus.	>100	43 (29-57)	30 (19-48)	28 (17-44)	14 (11-23)	14 (11-23)
Crayfish (14 days old)						
Orconectes nais.....	>100	34 (21-47)	20 (12-32)	20 (12-32)	20 (12-32)	20 (12-32)

[1]Hard water (pH, 7.2-7.4; total hardness 270 mg/l as $CaCO_3$) at 21 C.
[2]Values based on whole formulation rather than active ingredient.

Table 3. Uptake and magnification of ^{14}C-TFM by six aquatic invertebrates

Organism	Organisms[1] per sample	Water[2] concentration (mg/l)	Whole body residues (mg/kg) and magnification factor[3]				
			1 day	4 days	7 days	14 days	21 days
Scud (mature)..... *Gammarus pseudolimnaeus*	3	0.013	0.390 (30)	0.728 (56)	0.754 (58)	0.754 (58)	--
Scud (mature)..... *Gammarus pseudolimnaeus*	3	0.026	0.286 (11)	0.702 (27)	1.35 (51)	1.33 (51)	1.35 (52)
Scud (mature)..... *Gammarus pseudolimnaeus*	3	0.510	8.45 (16)	22.1 (43)	28.6 (56)	45.7 (89)	--
Waterflea (mature)..... *Daphnia magna*	60	0.026	0.120 (5)	0.136 (5)	--	--	--
Mayfly (early instar)..... *Stenonema sp.*	6	0.026	0.0360 (1.3)	0.056 (2.2)	0.110 (4.4)	--	--
Damselfly (early instar)... *Ischnura verticalis*	3	0.510	0.15 (0)	0.63 (1.2)	0.49 (1)	--	--
Crayfish (immature, 21 days)..... *Orconectes nais*	2	0.026	0.0360 (1.3)	0.048 (1.8)	0.052 (2)	0.068 (2.6)	0.068 (2.6)
Sowbug (mature)..... *Asellus brevicaudus*	4	0.020	0.28 (14)	0.74 (37)	--	--	--

[1]Samples were taken in triplicate.
[2]Hard water (pH 7.2 and total hardness 270 mg/l as $CaCO_3$) at 21 C.
[3]Concentration in organism (wet weight)/concentration in water.

Dissipation study

The dissipation of TFM residues by scud was determined by exposing them to 0.026 mg/l of ^{14}C-TFM for 7 days. This exposure was sufficient to induce a residue plateau. Once this plateau was reached, the scuds were transferred to TFM-free flowing water. The time required for 50% elimination of TFM by scud was 3.5 days. At 14 days, 98% of the radioactive residues had been lost. Analytical techniques to determine TFM degradation products in invertebrates are not well defined but it is assumed that the loss of radioactivity was due to excretion of TFM and/or metabolites of TFM.

Reproduction study

Continuous exposure of daphnids for three generations (63 days) to 2.4, 4.9, and 10 mg/l of field grade TFM (35.7% active ingredient) did not significantly impair reproduction when compared with controls. However, daphnids exposed at 18 mg/l of field grade TFM formed ephippial eggs (fertilized eggs) and reproduction stopped within the first generation (21 days).

DISCUSSION

Of the various chemicals toxic to sea lamprey, TFM is considered the most desirable for use in Great Lakes tributaries

because of its effectiveness as a lamprey larvicide and its safety to resident fish populations. Concentrations of 2-4 mg/l of TFM required to kill lamprey larvae in streams (Applegate et al. 1958) are not toxic to most aquatic invertebrates during acute exposures (Smith 1967; Erkkila 1962). Observations in the field have also shown that fish and reptiles (Applegate et al. 1961), and amphibians (Johnson 1959) are not affected by these TFM treatments.

Comparable data on the toxicities of TFM to aquatic invertebrates are limited to a few animals because many investigators have not included information on water quality or grade of TFM used in their experiments. Our LC50 values for scud and crayfish are in agreement with those of Erkkila (1962) and Smith (1967) who reported 24-h LC50 values of greater than 20 mg/l for both invertebrates. Applegate et al. (1958) found that the toxic effects of mononitrophenols on fish were considerably less under conditions that simulated treatment of an actual stream than effects which were observed under static conditions. Similar observations were noted in our studies, in which TFM was twice as toxic to crayfish in static toxicity tests than in flow-through tests.

Toxic chemicals introduced into the aquatic environment are often below levels acutely toxic to invertebrates. These sublethal concentrations, however, may impair successful growth, molting, and reproduction in invertebrate populations. Therefore, populations of fishes may be threatened because of loss of the fish-food organisms. The results from our reproductive toxicity tests indicate no reproductive impairment in D. magna when they were exposed continuously for three successive generations (63 days) to 10 mg/l of field grade TFM. Because of material cost, the continuous application of TFM in the aquatic environment never exceeds 24 h (Applegate and King 1962). Therefore, it seems highly improbable that daphnids would ever be exposed to concentrations greater than 10 mg/l, especially for 63 consecutive days.

Our knowledge of the metabolic fate of TFM in fish and invertebrates is incomplete. Lech (1971) found that TFM was degraded in rats to 3-trifluoromethyl-4-aminophenol (RTFM). He also found that TFM and RTFM were excreted in the urine as polar derivatives, some of which appear to be glucuronides. Although we only determined the loss of ^{14}C-TFM residues in scud, our results suggest that the dissipation of these residues was rapid. Therefore, significant TFM concentrations would not be expected to accumulate in top level consumers. Further studies, however, are needed to determine residue data from various components of simulated or natural intact food chains. The method of application, proper formulation, water quality characteristics, and species of animals in the area of TFM application are important factors to be considered in minimizing the hazard of TFM to aquatic animals.

SUMMARY

The acute toxicities of field grade TFM (35.7%) and purified TFM (95.7%) were determined for scud and crayfish in well water (pH 7.2 and total hardness 270 mg/l) at 21 C. The 96-h LC50 of field grade TFM was 57 mg/l for scud and 110 mg/l for crayfish; purified TFM was twice as toxic. The 30-day LC50 of field grade TFM was 14.3 mg/l for scud and 20.1 mg/l for crayfish.

Six species of aquatic invertebrates exposed to ^{14}C-TFM accumulated residues up to 58 times (wet weight) the concentration in water.

The amount of TFM accumulated by scud at equilibrium (7 days) was proportional to the concentration in water. However, magnification factors were relatively independent of water concentrations. When scud were transferred to TFM-free flowing water after 7 days of exposure to 0.026 mg/l of TFM, the residues decreased at 14 days from 0.754 mg/kg to 0.03 mg/kg.

Concentrations of 2.4, 4.9, and 10 mg/l of field grade TFM did not significantly impair reproduction in daphnids after 63 days of exposure. Total production of young was inhibited in 21 days at a concentration of 18 mg/l.

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Fish Control Laboratories
Fish and Wildlife Service
U.S. Department of the Interior
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